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14. ABSTRACT

The prevalence of breast cancer is greatest in industrialized regions and exposure to light at night has been proposed as a potential risk factor. Modulation of melatonin secretion by light has been implicated in the causal pathway linking exposure to light and breast cancer risk. Recent evidence indicates that melatonin is a natural oncostatic agent capable of functioning through a variety of anti-proliferative, anti-oxidative, and immunostimulatory mechanisms. We conducted a study to investigate the association of prediagnostic melatonin production and subsequent breast cancer risk in a prospective cohort study, the Italian ORDET study. Thus, prediagnostic melatonin production was measured as urine levels of the 6-hydroxymelatonin sulphate (6-OHMS), its primary enzymatic metabolite, in 12-hour urine (overnight) collection. The study was conducted as a nested case-control study. We included XXX breast cancer cases among cohort members during the 17 year-follow-up period. Four controls were matched to each case on age, menopausal status, recruitment center and time of recruitment.

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Breast cancer, Melatonin, Epidemiological Study

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TABLE OF CONTENTS

INTRODUCTION

Melatonin (N-acetyl-5methoxtryptamine) is synthesized and released by the pineal gland in response to darkness. Thus, melatonin displays a strong variation during a 24-hour period: its serum levels are low during daylight hours and high at night. The health effect of chronic alteration of this circadian rhythm in humans has received relatively little attention. There is strong evidence to indicate that melatonin acts as a natural oncostatic substance (Blansk, 1993). Consistent experimental evidence, from both *in vitro* and *in vivo* studies, identified specific anti-carcinogenic functions of melatonin such as anti-proliferation, anti-oxidation, and immunostimulation functions (Brzezinski, 1997; Panzer and Viljoen, 1997; Reiter et al, 1997).

Environmental factors that reduce nocturnal exposure to melatonin may increase breast cancer risk by increasing levels of estrogens, by increasing exposure to oxidative stress and by reducing immune function (Cohen et al, 1978; Stevens, 1987). Nighttime plasma melatonin is reported to be lower in women affected with estrogen-receptor-positive breast cancer in comparison with women affected by other pathologies (Tamarkin et al, 1989). Melatonin was also lower in breast cancer cases than in women with benign breast disease (Bartsh et al, 1989). We have conducted a study to evaluate the relationship between melatonin and breast cancer using data from a prospective cohort study in which several sources of possible biomarker variability have been controlled by study design. We measured pre-diagnostic urine levels of the main melatonin metabolite, **6-OHMS**, in urine stored at –80º C during the 17 year follow-up period. The study has allowed us to investigate the role of prediagnostic melatonin as a potentially important factor underlying the association between environmental and life-style factors with breast cancer.

BODY OF REPORT

In accordance with the Statement of Work we have completed all determinations for the samples from post-menopausal women and pre-menopausal women in the final year of the study. We have also completed analysis of the data on the post-menopausal cases and the results will be published in the June issue of JNCI. We are currently completing data analysis on the pre-menopausal cases and have preliminary results. The final results will be presented in a paper in 2 months.

Background

The Lombardy Cancer Registry (LCR) conducts the follow-up of the ORDET cohort. LCR, established by the Regional County Council for Health and supervised by the Epidemiology Unit at the National Cancer Institute in Milan, has been operating since January 1, 1976. LCR registers and includes in the incidence figures all malignant tumors, according to the categories 140-208, chapter two of the International Classification of diseases, ninth revision (ICD-9). The ORDET study and LCR reside in the same institution at the Italian NCI in Milan (Instituto Nazionale per lo Studio e la Cura dei Tumori). The LCR searches for cases actively, using various information sources, primarily hospital clinical records and pathology department records. The Italian National Health Service (NHS) provides health assistance for all citizens. Most health care services are public. Private facilities are also partially supported by NHS. Among all breast cancer cases arisen among residents of Varese Province, only 1.3% is known to the registry on the basis of death certificates only, and 99% of breast cancer cases are microscopically verified. LCR incidence data are regularly published in the "Cancer Incidence in Five Continents" (International Agency for Cancer Research-World Health Organization, 1982-1997; 1998-2002) and in several international publications on population-based cancer survivals (Eurocare Study I, 1995; Eurocare Study II, 1999). In the context of these studies on cancer survival, the LCR collects clinical information of cancer cases. Among this information, LCR collects the receptor status of the breast cancers identified in the population. This variable is included in the data analysis of the present study.

The end of follow-up is determined by death, immigration outside Italy or last day of the follow-up: in the case of the present application the last day of the follow-up was June 30, 2006. The latency period between cancer diagnosis and detection by the LCR is 6 months.

In the final year of the study we conducted the melatonin determinations for each identified case and the four related controls, after study protocols had been developed and discussed. Determinations having already been completed for samples from post-menopausal women the previous year, we then completed those for pre-menopausal women in the final year. Analysis of the data for post-menopausal women has already been completed and the findings published (see article attached). Statistical analysis for pre-menopausal women is currently being carried out and preliminary results will be followed by presentation of the full results in a paper in 2 months time.

Methods

Breast Cancer Cases: Breast cancer cases were women with histologically confirmed invasive breast cancer diagnosed after their recruitment (date at interview) to the ORDET cohort and before the end of the last follow-up period.

Control Subjects: Eligible controls were all women free of cancer at the time of the diagnosis of the case. For each breast cancer case, four controls were randomly chosen after matching for sources of hormone variability: a) age; b) same recruitment center to exclude differences due to transportation of samples to laboratory; c) recruitment date to control for the effect of long-term preservation of sera; d) daylight saving period to allow for possible changes in circadian rhythm.

12 Hour Urine Collection: For urine collection at baseline, each participant was asked to empty her bladder before retiring at 7:00PM, and to collect any urine voided during the night, as well as the first morning void at 7:00AM. Participants then delivered urine between 7:30 and 9:30AM to the ORDET recruitment center, where it was filtered and stored at –80°C. Urine samples were not thawed prior to determinations. Therefore,

there were no effects of freezing-thawing cycles and urine for this study was thawed at the time of the 6-OHMS determinations.

Analytical Methods: Melatonin production at baseline was evaluated through the urine excretion of 6-OHMS, its primary enzymatic metabolite using radioimmunoassay method (Bühlmann Laboratories AG, Switzerland). We corrected concentration levels of 6-OHMS for creatinine excretion. There is evidence that total nocturnal production of melatonin is well correlated with levels of 6-OHMS in 24 hour urine samples and with morning urine samples (Markey et al, 1985 and Bojkowski et al., 1987; Cook et al., 2000). 6-OHMS shows good reliability and low intra-individual variability, at least over a short time period (Bojkowski et al., 1987), reflecting a stable rate of melatonin production in the same individual (Bojkowski et al., 1987; Arendt J, 1978). Finally, 6-OHMS is extremely stable in urine stored at -20˚C and at -12˚C for at least two years of cryopreservation (Bojkowski et al., 1987).

Biological specimens of all cases and matched controls were retrieved from the ORDET biological specimen bank and sent, on dry ice, to the Hormone Research Laboratory, at the Department of Preventive and Predictive Medicine of the Istituto Nazionale Tumori under the direction of Dr. Giorgio Secreto. The Laboratory is located in the same building as the ORDET specimen bank. Stored samples from cases and controls were handled identically and assayed together in the same batch. Each batch included cases and their matched controls. Laboratory personnel were blinded to case control status of samples. In addition, we included blind control duplicates for 5% of the samples in each batch. All samples were assayed in duplicate.

The analytical determinations for all the biomarkers were completed in the final year of the study. Analysis on data from post-menopausal women was also carried out and the findings are going to be published (JNCI, in press). At the end of the study we are currently carrying out analysis from pre-menopausal women and plan to publish a paper on these findings.

Key Research Accomplishments

- Melatonin determinations for cases and controls were completed in the final year of the study.
- Data analysis was carried out on cases and controls for post-menopausal women.
- Results on post-menopausal cases were presented in a paper.
- Analysis of data on pre-menopausal women has begun and will be completed within the next 2 months. These results will then be presented in a paper, and at the “Era of Hope” meeting.

REPORTABLE OUTCOMES

All analysis of post-menopausal cases and related controls have been carried out. In total, 178 post-menopausal case patients with invasive or in situ breast cancer and 710 matched control subjects were available for our analyses. The analysis of the data found that, in conditional logistic regression models, the multivariate relative risk [reported as the odds ratio (OR)] of invasive breast cancer for women in the highest quartile of total overnight aMT6s output compared with the lowest was 0.56 [95% confidence interval (CI) = 0.33 to 0.97; Ptrend = 0.02]. This association was strongest among current non-smokers, excluding 28 cases who reported smoking cigarettes at the time of urine collection (OR, 0.38, 95% CI, 0.20-0.74; Ptrend = 0.001). Overnight urinary aMT6s level and breast cancer risk were more strongly associated in women who were diagnosed with invasive breast cancer more than 4 years after urine collection, compared to their controls (OR for highest versus lowest quartile of urinary aMT6s output = 0.34, 95% CI = 0.15 to 0.75). We did not observe important variations in relative risks by hormone receptor status of breast tumors. Please see the attached paper for further analysis.

Publications and Presentations

At the beginning of 2008 we published a paper based on the results of data analysis on postmenopausal women.

Urinary 6-Sulphatoxymelatatonin levels and risk of breast cancer in postmenopausal women: The ORDET cohort

Eva S. Schernhammer, Franco Berrino, Vittorio Krogh, Giorgio Secreto, Andrea Micheli, Elisabetta Venturelli, Sabina Sieri, Christopher T. Sempos, Adalberto Cavalleri, Holger J Schünemann, MD, Sabrina Strano, Paola Muti

Results of the data on pre-menopausal women will be presented in a paper this year and at the "Era of Hope" 2008 meeting.

In 2006 we published a paper based on the research developed during the evaluation of the bioassay method reliability:

Barba M, Cavalleri A, Schünemann HJ, Krogh V, Evangelista A, Secreto G, Micheli A, Zhou Q, Fuhrman B, Teter B, Berrno F, *Muti P.* **Reliability of urinary 6-sulfatoxymelatonin as a biomarker in breast cancer.** ***The International Journal of Biological Markers*****. *21(4):242-5, 2006***

In 2007-2008, Dr. Muti has published other papers on hormones and cancer, as listed below:

1) Browne R, Koury S, Marion S, Wilding G, **Muti P**, Trevisan M.. Accuracy and biological variation of human serum paraoxonase 1 activity and polymorphism (Q192R) by kinetic enzyme assay. Clin. Chem. Feb;53(2):310-7, 2007.

2) McCann SE, Wactawski-Wende J, Olson J, Ovando B, Nowell S, Davis W, Carter L, Muti P, Shields PG, Freudenheim JL, **Muti P**. Changes in 2-hydroxyestrone and 16alpha-hydroxyestrone metabolism with flaxseed consumption: modification by

COMT and CYP1B1 genotype. Cancer Epidemiology Biomarkers and Prevention 16(2):256-62, 2007

3) McCann SE, McCann WE, Hong C, Marshall JR, Edge S, Trevisan M, **Muti P**, Freudenheim JL. Dietary patterns related to glycemic index and load and risk of pre- and postmenopausal breast cancer in the Western New York Exposure and Breast Cancer Study. American Journal of Clinical Nutrition 86(2):465-71, 2007

4) Sieri S, Pala V, Brighenti F, Pellegrini N, **Muti P**, Micheli A, Evangelista A, Grioni S, Contiero P, Berrino F and Krogh V. Dietary glycemic index, glycemic load and risk of breast cancer in the ORDET cohort. The American Journal of Clinical Nutrition 86(4):1160-6, 2007

5) Sant M, Allemani C, Sieri S, Krogh V, Menard S, Tagliabue E, Nardini E, Micheli A, Crosignani P, **Muti P**, Berrino F. Salad vegetables dietary pattern protects against HER-2-positive breast cancer: A prospective Italian study. Int J Cancer. 121(4):911-914, 2007

6) Schunemann HJ, Castell M, **Muti P**. Systematic reviews for the Journal of Experimental and Clinical Cancer Research: Going where the science takes us. J. Exp. Clin. Cancer. Res.26(2);169-174, 2007

7) Nie J, Beyea J, Bonne MR, Han D, Vena J, Rogerson P,Vito D, **Muti P**, Trevisan M, Edge S, Freudenheim JL. Exposure to Traffic emissions throughout life and risk of breast cancer: the Western New York exposures and breast cancer (WEB) study. Cancer Causes Control 18(9):947-55

8) Gaikwad NW, Yang L, **Muti P**, Meza JL, Pruthi S, Ingle JN, Rogan EG, Cavalieri EL The molecular etiology of breast cancer: Evidence from biomarkers of risk (Int J Cancer) 2007 Dec. 20

9) Fuhrman BJ, Teter BE, Barba M, Byrne C, Cavalleri A, Grant BJ, Horvath PJ, Morelli D, Venturelli E, **Muti P**. Equol status modifies the association of soy intake and mammographic density in a sample of postmenopausal women. Cancer Epidemiology, Biomarkers & Prevention. 2008 Jan;17 (1): 33-42)

10) Akl E, Barba M, Rohilla S, Terrenato I, Sperati F, **Muti P**, Schünemann HJ. Anticoagulation for the long term treatment of venous thromboembolism in patients with cancer. (in press, BMJ)

11) Capurso G, Schünemann HJ, Terrenato I, Morretti A, Koch M, **Muti P**, Capurso L, Delle Fave G. Meta-analysis: the use of non-steroidal anti-inflammatory drugs and pancreatic cancer risk for different exposure categories. Aliment Pharm & Therapy 2007 Oct;26(8): 1089-99

12) Akl EA, Rohilla S, Barba M, Sperati F, Terrenato I, Muti P, Shunemann HJ. Anticoagulation for the initial treatment of venous thromboembolism in patients

with cancer. Cochrane Database Syst Rev. 2008 Jan 23;(1): CD006649. Review. PMID: 18254108

13) Barba M, Terrenato I, Schünemann H, Fuhrman B, Sperati F, Teter B, Gallucci M, D'Amato A, **Muti P**. Indicators of sexual and somatic development and adolescent body size in relation to prostate cancer risk: results form a case control study. Urology. 2008 Feb 14: (Epub ahead of print)

14) Akl EA, Terrenato I, Barba M, Sperati F, Sempos HV, **Muti P**, Cook DJ, Schunemann HJ. Low molecular weight heparin versus unfractionated heparin for perioperative thromboprophylaxis in patients with cancer: a systematic review and a meta-analysis (in press, Archives of Internal Medicine)

15) Akl EA, Rohilla S, Barba M, Sperati F, Terrentao I, **Muti P**, Bdair F, Schunemann HJ Anticoagulation for the initial treatment of venous thromboembolism in patients with cancer: a systematic review (under review, Cancer, Journal of the American Cancer Society)

Presentations/Abstracts

1) Fuhrman BJ, Teter BE, Barba M, Byrne C, Cavalleri A, Grant BJ, Horvath PJ, Morelli D, Venturelli E, Muti P. Dietary Macronutrients and Equol Status as Determinants of Mammographic Density in a Sample of Postmenopausal Women from Western New York, USA. (poster presentation at the 2007 AACR annual meeting)

2) Teter BE, Fuhrman BJ, Barba M, **Muti P**. Dietary Antioxidants and Mammographic Breast Density as a Marker of Breast Cancer Risk in Postmenopausal Women**.** Poster presentation at the 2007 AACR annual meeting)

3) Crespo C, Garcia-Palmieri M, Smit E, Lee IM, McGee D, **Muti P**, Figueroa Valle N, Ramirez-Marrero F, Freudenheim J, Sorlie P. Physical activity and prostate cancer mortality in Puerto Rican men.

4) Meneghini E, Secreto G, Krogh V, Crosignani, **Muti P**, Berrino F, Micheli A. Biological adjustment approach in synchronizing blood sampling over menstrual cycle. The experience with progesterone and breast cancer risk in pre-menopausal ORDET women. Annual Meeting of the Cancer Registries of Latin Language (GRELL), Montreal, May 2007.

5) Akl E, Cook D, **Muti P**, Puhan M, Montori V, Guyatt G, Schünemann H. Systematic evaluation of the methodology of randomized controlled trials of anticoagulation in patients with cancer. 15th Cochrane Colloquium, São Paulo, 23-27 October, 2007.

6) Schernhammer E.S, Berrino F, Krogh V, Secreto G, Micheli A, Venturelli E, Sieri S, Sempos C.T, Cavalleri A, Schunemann H.J, Strano S, **Muti P**. Urinary 6-Sulphatoxymelatonin Levels and Risk of Breast Cancer in Postmenopausal Women: The Ordet Cohort. DF/HCC Breast Cancer Researchers' Retreat.Boston, USA,14 March 2008

7) Schernhammer E.S, Berrino F, Krogh V, Secreto G, Micheli A, Venturelli E, Sieri S, Sempos C.T, Cavalleri A, Schunemann H.J, Strano S, **Muti P**. *Urinary 6-Sulphatoxymelatonin Levels and Risk of Breast Cancer in Postmenopausal Women: The Ordet Cohort.* AACR 99th Annual Meeting, San Diego, USA 12-16 April 2008

8) Schernhammer E.S, Berrino F, Krogh V, Secreto G, Micheli A, Venturelli E, Sieri S, Sempos C.T, Cavalleri A, Schunemann H.J, Strano S, **Muti P**. Urinary 6-Sulphatoxymelatonin Levels and Risk of Breast Cancer in Postmenopausal Women: The Ordet Cohort. Era of Hope 2008 Meeting. Baltimore, USA 25-28 June 2008 (abstract submitted)

*Presentations by students and postdoctoral fellows under the supervision of Dr. Muti

In addition, Dr. Muti has several other manuscripts submitted for publication on hormone and related factors and cancer.

CONCLUSIONS

In summary, our findings suggest that melatonin secretion, as assessed by aMT6s levels in 12-hour overnight urine, plays an important role in postmenopausal breast cancer development. They also indicate that factors that affect melatonin's metabolization must be carefully taken into account when using this marker. To our knowledge, these prospective data provide, for the first time, evidence for a significant, inverse association between melatonin levels, as measured in overnight morning urine, and breast cancer risk in postmenopausal women, and thus represent an important step forward in our understanding of this relationship. Studies to confirm our findings should address how melatonin levels measured in 24-hour urine samples differ from those measured in 12-hour overnight or first-morning urine and through which primary mechanisms, if not via the gonadal axis or tumor hormone receptors, melatonin affects breast cancer risk.

When we have the final analysis we expect this preliminary data to also be confirmed in pre-menopausal women. We will then prepare and publish a paper on the related study.

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URINARY 6-SULPHATOXYMELATONIN LEVELS AND RISK OF BREAST CANCER IN POSTMENOPAUSAL WOMEN: THE ORDET COHORT

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ABSTRACT

Background. Several factors including light at night, age, and body mass index appear to influence melatonin production. Lower urinary melatonin levels have been associated with a higher risk of breast cancer in premenopausal women. The association between melatonin levels and breast cancer risk in postmenopausal women remains unclear.

Methods. In a prospective case–control study nested within the ORDET, we measured the concentration of melatonin's major metabolite, 6-sulphatoxymelatonin (aMT6s), in the 12-hour overnight urine of 178 postmenopausal women with incident breast cancer and 710 matched control subjects.

Results. In conditional logistic regression models, the multivariate relative risk [reported as the odds ratio (OR)] of invasive breast cancer for women in the highest quartile of total overnight aMT6s output compared with the lowest was 0.56 [95% confidence interval (CI) = 0.33 to 0.97; P_{trend} = 0.02]. This association was strongest among current non-smokers, excluding 28 cases who reported smoking cigarettes at the time of urine collection (OR, 0.38, 95% CI, 0.20-0.74; P_{trend} = 0.001). Overnight urinary aMT6s level and breast cancer risk were more strongly associated in women who were diagnosed with invasive breast cancer more than 4 years after urine collection, compared to their controls (OR for highest versus lowest quartile of urinary aMT6s output = 0.34, 95% CI = 0.15 to 0.75). We did not observe important variations in relative risks by hormone receptor status of breast tumors.

Conclusion. These prospective data provide, to our knowledge for the first time, evidence for a significant, inverse association between melatonin levels, as measured in overnight morning urine, and breast cancer risk in postmenopausal women.

Introduction

Many biologic functions in humans follow distinct, approximately 24-hour patterns (1) that are driven by a circadian pacemaker located in the hypothalamus (2). Secretion of melatonin, an indoleamine hormone that is produced primarily by the pineal gland, also follows a circadian rhythm of approximately 24 hours; melatonin is secreted exclusively during the dark phase of a light–dark cycle (3). The urine concentration of the major metabolite of melatonin, 6-sulphatoxymelatonin (aMT6s), is highly correlated with melatonin levels in blood and saliva (4-10). Urinary aMT6s levels, as measured in first morning urine, accurately reflect plasma melatonin levels measured during the previous night (5, 11).

Results of previous studies [reviewed in (12)] suggest that night-shift work, a surrogate for exposure to light at night, is associated with an increased risk of breast cancer. On the basis of results of laboratory and animal experiments (13, 14), light-induced suppression of melatonin secretion has been hypothesized as the major cause of this association; however, the only two prospective studies conducted to date to study associations between circulating melatonin and breast cancer risk were among premenopausal women, and results were inconsistent: one study found no evidence that 24-hour urinary levels of melatonin are strongly associated with the risk of breast cancer (15), whereas the other study reported their first morning urinary levels of melatonin to be strongly and inversely related to risk of breast cancer (16). No prior study has evaluated this association in postmenopausal women. Uncertainty exists about the optimal urine sampling procedure to best capture nightly melatonin.

We used a nested case–control design to conduct a prospective study of the association between melatonin levels in 12-hour overnight urine and breast cancer risk in a large cohort of postmenopausal women enrolled in ORDET. We evaluated associations between total aMT6s

produced between 7:00pm and 7:00am and creatinine-adjusted aMT6s measured in overnight urine and postmenopausal breast cancer risk.

Methods

The *Hormones and Diet in the Etiology of Breast Cancer Risk* (ORDET) cohort was established in northern Italy between June 1987 and June 1992, when 10,786 healthy women ages 35 to 69 years, were enrolled (17, 18). They were all residents of the Varese province, — an area covered by the Lombardy Cancer Registry (19), who had heard about the study through the media, at public meetings, and at breast cancer early detection centers and who volunteered to participate. At recruitment, a number of baseline characteristics including demographics and dietary intake were queried from each participant via questionnaire, direct measurements of several anthropometric variables including height and weight were conducted, and blood and urine specimens were collected. Because of the focus of the study on endogenous hormones and their relationship with breast cancer risk, stringent inclusion criteria were established and highly standardized conditions on collecting biological samples were applied. Women were excluded if they reported a bilateral ovariectomy, were currently breast feeding or pregnant, used oral contraceptives or hormone replacement therapy in the last three months, were affected by metabolic diseases, or reported a history of cancer.

Cancer incidence information, available from the local cancer registry (Varese Cancer Registry) was linked to the ORDET cohort in order to identify incident breast cancer cases up to December 2003. The Varese Cancer Registry is of high quality: < 2% of breast cancer cases are known to the registry by death certificate only, and the histology and cytology of 96.3% of all

cases has been confirmed through pathology reports (17, 20). The ORDET file was also linked to the Varese residents' file to check participants' vital status.

After exclusion of women with a history of cancer (with the exception of non-melanoma skin cancer) and women who, immediately after baseline, were lost to follow-up (observed time=0), 10,633 participants remained to form the base population of ORDET. For this study, we further restricted the ORDET cohort to its 3,966 postmenopausal participants. Women were considered postmenopausal when they reported not having any menstrual cycle over the past twelve months. Participants were censored at the time of cancer diagnosis, death, or loss to follow-up, whichever came first (median follow-up time, 13.5 years).

Selection of Case and Control Subjects

Case and control subjects were selected from among all 3,966 eligible postmenopausal women. Case subjects were women who developed breast cancer after their recruitment into the ORDET cohort but before the end of the study period (December 31, 2003). We identified a total of 184 incident breast cancer cases. Of these, three women were eliminated because breast cancer was not their first cancer and another three women because no urine was collected. Of the remaining 178 cases, 7 had in situ breast cancer.

For each case subject with breast cancer, four control subjects were randomly chosen from among appropriate risk sets consisting of all cohort members who satisfied the matching criteria and were alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. An incidence density sampling protocol for control selection was used, such that controls could include subjects who became a case later (14 women), while each control subject could also be

sampled more than once (42 women). Matching characteristics were age (± 3 years) at enrollment, date of recruitment (± 180 days), and laboratory batch. A total of 710 controls were selected.

Specimen collection

Women were instructed to collect their urine over the previous night. They followed a collection protocol which called for discarding the last void at 7:00pm and collecting urine during the night up to 7:00am. Overnight urine was kept at room temperature during collection. After delivery to the ORDET recruitment center the day after overnight collection between 7:30am and 9:00am, all urine samples were immediately processed and stored at –80 degrees Celsius until biochemical determinations were done. Urine was filtered and separated, and 6ml aliquots were stored. No preservatives were added either at collection or during storage. Similarly, blood samples were collected after at least 12 hours of fasting between 7:30am and 9:00am and stored at –80 degrees Celsius. This study was approved by the Ethical Review Board of the National Cancer Institute of Milan (Italy).

Laboratory Methods

Stability and reliability of the ORDET collection method for aMT6s have been demonstrated (21) to be reasonable, although storage temperature affected specimens such as that urine stored long-term at –30 degrees Celsius had systematically lower aMT6-s levels than urine stored at –80 degrees Celsius.

Urine samples from breast cancer cases and related controls were handled identically and assayed together on the same day and in the same run. All samples were taken out of the freezer simultaneously and sent to laboratory in the same parcel on dry ice. They were stored at –80

degrees C for an average of 17 years. Laboratory personnel were blinded to case-control status. Control of analytic error was based on the inclusion of two standard samples.

Urinary aMT6s was assayed by the Hormone Research Laboratory, Etiological Epidemiology and Prevention Unit, at the National Cancer Institute Foundation (Milan, Italy), using the Bühlmann enzyme-linked immunosorbent assay EK-M6S (Bühlmann Laboratories AG, Allschil, Switzerland) with a lower detection limit of 0.8 ng/mL for aMT6s. Briefly, prediluted (1:200) urine samples, ready-to-use calibrators and controls, and biotinylated aMT6s competes with the aMT6s present in the sample for the binding sites of an anti-aMT6s antibody to form a complex which is captured by a second antibody coated on the wells. The addition of the enzyme label (streptavidin conjugated to horseradish peroxidase) and, subsequently, of the tetramethylbenzidine substrate generates a blue-colored product, the amount of which is inversely proportional to the quantity of aMT6s originally present in the sample. Adding acidic stop solution turns the color from blue to yellow. The color intensity is measured at a wavelength of 450 nmol in a microtiter plate reader in order to plot a standard curve and to calculate the aMT6s concentration.

Creatinine levels were also measured for each sample by the Medical Laboratory of the Department of Oncology, National Cancer Institute Foundation (Milan, Italy), with a Hitachi Modular Automatic Analyzer and optimised reagents (F. Hoffmann-La Roche Ltd, Basel, Switzerland) (22). The average between-batch coefficient of variation was 5.3% and 10.3% for urinary aMT6s (high and low standard QCs), and 2.7% and 2.1% for creatinine concentrations of 1.2 mg/dl and 4.37 mg/dl, respectively. The within-batch CVs derived from quality control urine included in the analytic runs were 1.8% and 9.8% for aMT6s (high and low standard QCs).

Plasma sex steroid measurements (testosterone, free testosterone, SHGB, and estradiol) were conducted by Centro Medico Diagnostico Emilia (Bolgona, Italy). For testosterone and free testosterone, we used Coat-A-Count procedure, a solid-phase radioimmunoassay (Diagnostic Product Corporation, Los Angeles, USA); for SHGB, IMMUNOLITE 1000 Analyzer, a solid-phase, chemiluminescent immunometric assay (Diagnostic Product Corporation, Los Angeles, USA); and for estradiol, Orion Diagnostica SPECTRIA Estradiol *Sensitive* RIA test, a coated tube radioimmunoassay (Orion Diagnostica Oy, Espoo, Finland). The between-batch CVs derived from quality controls included in the analytic runs ranged from 5.8% to 18.3% for all four analytes (high and low standard QCs).

Statistical analyses

In total, 178 case patients with invasive or in situ breast cancer and 710 matched control subjects were available for our analyses. Nine women had aMT6s levels that were below the limit of detection for the assay (i.e., <0.8 ng/mL) and their values were calculated by extrapolation on the standard curve. We multiplied aMT6s concentration (ng/ml) with 12-hour urine volume to obtain total aMT6s produced between 7:00pm and 7:00am (reported as μg per 12 hours). In secondary analyses, aMT6s levels were normalized to the creatinine level of the sample to account for differences arising from variations in urine concentrations (reported as ng aMT6s per mg of creatinine).

To test for differences in hormone levels between case and control subjects, we used mixed-effects regression models for clustered data to adjust for possible confounding due to the matching factors and for any residual correlation between case and control subjects within the matched set (23). We used conditional regression models to estimate the relative risks of breast

cancer [reported as odds ratios (ORs) with 95% confidence intervals (CIs)] by quartiles of urinary aMT6s concentrations, which were defined on the basis of the values for all control subjects. Multivariate models were adjusted for the case–control matching factors as well as for known risk factors for breast cancer [see foot note to Table 3]. We tested for trends by modeling natural aMT6s concentrations continuously and calculating the Wald statistic. To evaluate the presence of an interaction between smoking (binary; current versus past or never smokers) and circulating aMT6s levels (continuously), we added an interaction term into our logistic regression model and used the likelihood ratio test for interaction to determine significance. We used SAS version 9.1.3 (Cary, NC) for all analyses. All *P* values were two-sided.

Results

Table 1 shows baseline characteristics of the 178 cases and 710 controls. The mean time between urine collection and diagnosis was 80 months (SD 50) with a range of 1-182 months. Study participants were all postmenopausal with an age range of 41 to 70 years at urine collection. Most of the women's baseline characteristics did not differ by case–control status (Table 1). However, age-adjusted mean urinary aMT6s concentration of the breast cancer cases was slightly lower than that of controls [11.1 µg aMT6s versus 12.1 µg aMT6s; 21.0 ng aMT6s/mg creatinine versus 23.5 ng aMT6s/mg creatinine]. Table 2 shows age and age-adjusted baseline characteristics by quartiles of urinary overnight aMT6s (µg) among the 710 controls included in this study. Several of the women's baseline characteristics, including age, family history of breast cancer, history of benign breast disease, smoking, and BMI, differ modestly by aMT6s quartile (Table 2). From among several sex steroids, including circulating plasma testosterone, free testosterone, SHBG, and estradiol, none appeared to vary substantially by aMT6s level.

Overall, we observed an inverse association between urinary aMT6s concentrations and breast cancer risk (OR for highest versus lowest quartile of urinary aMT6s concentration, 0.68; 95% CI, 0.42 to 1.11; P_{trend} = 0.09; Table 3), with little change in these estimates after additional adjustment for breast cancer risk factors including current smoking status. Night work and melatonin have been more strongly related to invasive than in situ breast cancer risk (16, 24), and we, therefore, excluded seven cases who were diagnosed with in situ breast cancer and their matched controls. Among women with invasive breast cancer only, the inverse association was slightly stronger (multivariate OR for highest versus lowest quartile of urinary aMT6s concentration, 0.59; 95% CI, 0.35 to 1.00; P_{trend} = 0.04).

When we evaluated the influence of sex steroid hormones on these associations, none of the hormones found to predict postmenopausal breast cancer risk was strongly correlated with urinary aMT6s (all Spearman rank correlations ≤0.15: r=0.05, p=0.20 for testosterone; r=-0.01, p=0.87 for free testosterone; r=0.02, p=0.62 for estradiol; and r=0.15, P<0.001 for SHBG;), and further adjustment for testosterone, free testosterone, estradiol, or SHBG did not alter our estimates substantially, although the OR did reach statistical significance after adjustment for circulating testosterone (OR, 0.56; 95% CI, 0.33-0.97; Table 3).

On the basis of a previous study suggesting that the nocturnal plasma melatonin increase inversely correlates with tumor estrogen receptor (ER) concentration (25), we conducted analyses stratified on ER status. For all breast cancer cases, hormone receptor status including HER2 status was available: 78.5% were ER+ tumors (only 38 women had ER– breast tumors), and 81.3% were HER2– (only 31 women had HER2+ breast tumors). When we restricted analysis to women with ER+ breast tumors, the inverse association between aMT6s and breast cancer risk remained by and large unchanged (multivariate OR for highest versus lowest quartile

of urinary aMT6s concentrations, 0.59; 95% CI, 0.30-1.13) and was virtually the same when we restricted to women with HER2– tumors (multivariate OR for highest versus lowest quartile of urinary aMT6s concentrations, 0.60; 95% CI, 0.32-1.13).

We found no effect modification by age (<59, ≥59 years old) or BMI (stratified along the median, 25.6). Because a previous study suggested that cigarette smoking affects melatonin production in premenopausal women (26), we further stratified by smoking status. Among never or past smokers, we observed a significant inverse association between urinary melatonin levels and breast cancer risk (highest versus lowest quartile of urinary aMT6s concentration, 0.38; 95% CI = 0.20 to 0.74; P_{trend} = 0.001; Table 3). By contrast, we did not observe an inverse association among women who reported cigarette smoking at the time of urine collection (highest versus lowest tertile of urinary aMT6s concentration, age-adjusted OR, 3.55; 95% CI, 0.61 to 20.8; P_{trend} = 0.07; χ^2 from LLH ratio test for interaction between smoking and aMT6s, 9.22, p (1df) = 0.002), although power was limited in these analyses with only 28 breast cancer cases among current smokers.

To exclude the possibility of preclinical tumors influencing our aMT6s levels, in subanalyses, we excluded cases that were diagnosed shortly after urine collection. In these analyses, the association between urinary aMT6s level and breast cancer risk became increasingly stronger after excluding case patients who were diagnosed with invasive breast cancer within 2 years (OR for highest versus lowest quartile of urinary aMT6s concentration and risk of breast cancer developed at least more than two years after urine collection, 0.35; 95% CI, 0.17 to 0.71; P_{trend} <0.001) or 4 years after urine collection (OR for highest versus lowest quartile of urinary aMT6s concentration, 0.34; 95% CI = 0.15 to 0.75; P_{trend} = 0.002).

Urinary creatinine concentration is influenced by a number of factors including gender, ethnicity, age and body-mass index (BMI) (27). While our study was exclusively comprised of Caucasian women, differences in age and BMI may have biased our creatinine-adjusted aMT6s measure. There was no correlation between creatinine-adjusted aMT6s and creatinine (Spearman r=-0.02, P=0.70), however, suggesting no major bias introduced by adjusting for creatinine. Therefore, in secondary analyses, we also evaluated associations between creatinine-adjusted aMT6s and breast cancer risk. Creatinine-adjusted and total aMT6s were highly correlated (Spearman r=0.93, P<0.001) and both measures also correlated well with crude aMT6s concentration (r=0.83, P<0.001). In multivariate analyses, we observed an inverse association between creatinine-adjusted urinary aMT6s and invasive breast cancer risk (OR for highest versus lowest quartile of total urinary aMT6s, 0.63; 95% CI, 0.37 to 1.07; P_{trend} = 0.02), a risk which was also markedly stronger among never and past smokers (highest versus lowest quartile of total 12-hour urinary aMT6s, 0.49; 95% CI = 0.26 to 0.91; P_{trend} = 0.003).

Discussion

We found a significant inverse association between overnight urinary aMT6s and breast cancer risk in postmenopausal women, a finding which was even stronger after the exclusion of current smokers. When using creatinine-adjusted aMT6s production as a measure for melatonin, our findings were similar. Ours is the first study to report on these associations.

Few prior studies have evaluated the association between circulating melatonin levels and breast cancer risk in humans and most are limited by the fact that melatonin levels were measured after the subjects were diagnosed with breast cancer (6, 11, 25, 28-36). The only two prospective studies to date were conducted among premenopausal women, and each of them

used a different measure of melatonin. The first study used 24-hour urine and found no association between circulating aMT6s and breast cancer risk (15). The other study, which used first spot morning urine to determine aMT6s, found a strong inverse association between their first morning urine aMT6s levels and premenopausal breast cancer risk (16). It has subsequently been argued that pooled urine samples collected over 24 hours cannot detect potential differences between subjects in the nocturnal duration or the peak of melatonin secretion, which may be important for assessing an association between melatonin levels and breast cancer risk (37).

What constitutes the best urine sample (e.g., 24-hour urine, 12-hour overnight urine, or first-morning voids) to reflect circulating levels of a hormone with substantial variation in secretion throughout the day remains subject of research. Spot urine samples are most practical, especially in large epidemiologic studies; a common approach to correct for varying dilutions among spot urine samples is to adjust for urinary creatinine (27). However, urinary creatinine concentration is influenced by a number of factors including gender, ethnicity, and age (27). Thus, the independence of effects of creatinine-adjustment has to be evaluated carefully in each individual study. In addition, just like 24-hour urine, a single untimed spot urine, is also likely unable to accurately capture the concentration of a hormone with varying secretion. Using timed 12-hour urine samples can perhaps overcome some of these limitations, but few studies exist that have examined differences between various sampling methods. For cortisol, studies seem to suggest that overnight urine measures, while comparable to 24-hour urine measures, require a larger sample size to find true effects (38, 39). Given the opposite physiologic 24-hour secretion rhythm of the two hormones (i.e., peak production in the morning and a nadir at night for cortisol; peak production at night and nadir during the day for melatonin), this perhaps suggests for melatonin that an overnight urine sample increases the likelihood of finding a true association

between melatonin and breast cancer risk, when compared to a 24-hour urine sample, though studies are needed that can contrast these various measures.

We were able to consider most important breast cancer risk factors in our analyses. Excluding cases diagnosed within the first 2 or 4 years after urine collection did not alter our findings; on the contrary, the risk reduction observed with higher aMT6s levels appeared slightly stronger in these analyses, suggesting that aMT6s levels are truly predictive of breast cancer risk and not simply a marker for tumor growth.

The interaction between smoking and aMT6s levels in our data is novel and highlights some of the additional challenges posed by using a marker that is measured in urine, making it highly dependent on its metabolization rate. CYP1A2 is the primary enzyme involved in the metabolization of melatonin to urinary aMT6s (40) and smoking has been shown to induce CYP1A2 activity (40, 41). Higher CYP1A2 activity, on the other hand, appears to be associated with breast cancer risk, particularly in postmenopausal women (42). The interaction between smoking and aMT6s levels in our sample could be indicative of the role of CYP1A2 activity in these associations and may ultimately suggest that smoking renders urinary aMT6s levels a less useful marker for breast cancer risk prediction, particularly in postmenopausal women. Total nocturnal plasma melatonin output and first-morning void urine 6-hydroxymelatonin sulfate (6-OHMS) in a group of 29 older women 40 to 70 years old was found to correlate well – however, whether smoking may have differentially affected these findings was not reported (6). In a comparable data set, no interaction between smoking and aMT6s levels was observed (unpublished data); yet aMT6s was measured in first morning urine in that study (16), as opposed to the overnight urine from our current data set. Clearly, more studies are needed to better understand the effect of smoking on circulating melatonin and its metabolization.

Our study is limited by the absence of information on light exposure at night including night work status, thus we cannot adjust for this factor. However, that risks were similar among the older women in our study (who were likely already retired at the time of urine collection), compared to younger women, alleviates some of that concern. Another potential limitation of our study is that we did not have information on vitamin D status in our study subjects, another possible breast cancer risk factor (43, 44). The relationship between melatonin levels and vitamin D is unclear, but if one exists it could have influenced our results. For example, it is conceivable that women with low morning melatonin levels (if due to an altered sleep-wake cycle) also have particularly low levels of vitamin D mediated by low sun exposure. In the current data set, we were unable to tease the two measures apart.

In summary, our findings suggest that melatonin secretion, as assessed by aMT6s levels in 12-hour overnight urine, plays an important role in postmenopausal breast cancer development. They also indicate that factors that affect melatonin's metabolization must be carefully taken into account when using this marker. Studies to confirm our findings should address how melatonin levels measured in 24-hour urine samples differ from those measured in 12-hour overnight or first-morning urine and through which primary mechanisms, if not via the gonadal axis or tumor hormone receptors, melatonin affects breast cancer risk.

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Table 1. Baseline characteristics[*] of 178 postmenopausal women with invasive (n=171) or in situ (n=7) breast cancer and their 710 matched controls.

All women[†]	**Cases (n=178)**	**Controls (n=710)**
Age, years	57.7 (5.9)	57.8 (5.6)
Urinary aMT6s, ng/ml creatinine	21.0 (1.10)	23.5 (0.55)
Urinary aMT6s/12 hrs, µg	11.1 (0.55)	12.1 (0.27)
Age at menarche, years	13.2 (1.6)	13.3 (1.6)
Age at menopause, years	48.8 (4.9)	48.6 (5.0)
Parity (among parous women only; %)	2.2 (1.1)	2.3 (1.1)
Age at first birth (among parous women only)	26.9 (4.2)	26.6 (4.4)
Family history of breast cancer (%)	12.9	7.2
HRT use (%)	19.1	16.8
Years of HRT use (among HRT users only)	2.5 (3.1)	1.6 (2.2)
OC use (%)	10.7	13.2
BMI, kg/m^2	26.2 (4.4)	26.1 (4.1)
Alcohol consumption, grams/day	11.3 (14.6)	10.4 (13.6)
History of benign breast disease (%)	9.6	8.2
Education beyond 8 years elementary school (%)	12.9	14.9
Smoking history		
Current smoker (%)	16.3	14.9
Past smoker (%)	11.2	11.5
Never smoker (%)	72.5	73.6
Pack-years among ever smokers	12.9 (12.3)	11.3 (11.0)
Sex hormone levels		
SHBG (nmol/L)	94.2 (3.86)	100.5 (1.94)
Testosterone (ng/ml)	0.32 (0.02)	0.26 (0.01)
Free testosterone (pg/ml)	0.75 (0.06)	0.61 (0.03)
Estradiol (pg/ml)	9.66 (1.73)	10.7 (0.91)

[*]Mean (SD).
[†] At natural menopause

Table 2. Age and age-adjusted baseline characteristics[*] of 710 controls by quartiles of urinary 6-sulfatoxymelatonin (aMT6s) level.

	Quartiles of 12-hour overnight urinary 6-sulfatoxymelatinin (aMT6s) output (µg)			
All control women[†]	**Q1 (n=177)**	**Q2 (n=178)**	**Q3 (n=178)**	**Q4 (n=177)**
Range of urinary aMT6s output/12 hours (µg)	<6.5	6.5-10.8	10.8-16.5	≥16.5
Age, years	59.2	57.3	58.1	56.0
Age at menarche, years	13.4	13.2	13.3	13.3
Age at menopause, years	48.4	49.0	48.8	48.4
Parity (# of children, among parous women only)	2.5	2.4	2.1	2.3
Age at first birth (among parous women only)	26.0	26.5	27.3	26.6
Family history of breast cancer (%)	5.1	6.2	7.3	10.1
HRT past use (%)	21.5	17.0	14.6	14.0
OC use (%)	12.1	15.8	16.4	9.7
BMI, kg/m^2	26.4	26.2	26.2	25.8
Alcohol consumption, grams/day	9.1	10.1	12.8	9.7
History of benign breast disease (%)	6.8	6.8	6.7	12.4
Education beyond 8 years elementary school (%)	9.0	16.4	15.2	19.1
Smoking history				
Current smoker (%)	16.9	16.8	12.0	12.5
Past smoker (%)	8.1	12.9	10.7	10.0
Never smoker (%)	75.0	70.3	77.3	77.5
Pack-years among ever smokers	13.6	10.2	10.4	9.3
Sex hormone levels				
SHBG (nmol/L)	97.4	99.8	95.8	109.2
Testosterone (ng/ml)	0.24	0.26	0.27	0.27
Free testosterone (pg/ml)	0.53	0.62	0.62	0.65
Estradiol (pg/ml)	10.3	11.1	9.6	11.7

[*]Mean.
[†] At natural menopause

Table 3. Odds ratios (ORs) and 95% confidence intervals of breast cancer by quartile of total 12-hour overnight 6-sulfatoxymelatonin (aMT6s) output (aMT6s concentration (ng/ml) *12-hour volume in ml).

	Quartile				
Group and parameter	**1**	**2**	**3**	**4**	P_{trend} ‡
Urinary aMT6s output/12 hours (µg)	<6.5	6.5-10.8	10.8-16.5	≥16.5	
No. of case patients/No. of control subjects	56/177	37/177	45/178	40/177	
Invasive and in situ breast cancer cases					
Simple OR*	1.00 (ref.)	0.65 (0.41-1.04)	0.79 (0.51-1.23)	0.68 (0.42-1.11)	0.09
Multivariate OR†	1.00 (ref.)	0.68 (0.42-1.11)	0.84 (0.53-1.35)	0.65 (0.39-1.09)	0.08
Invasive breast cancer cases					
Simple OR*	1.00 (ref.)	0.67 (0.42-1.07)	0.76 (0.48-1.20)	0.63 (0.39-1.04)	0.05
Multivariate OR†	1.00 (ref.)	0.70 (0.43-1.14)	0.82 (0.50-1.34)	0.59 (0.35-1.00)	0.04
Multivariate OR † adjusting for testosterone	1.00 (ref.)	0.69 (0.41-1.14)	0.84 (0.51-1.38)	0.56 (0.33-0.97)	0.02
Excluding current smokers					
No. of case patients/No. of control subjects	51/148	31/147	36/155	25/155	
Simple OR*	1.00 (ref.)	0.69 (0.41-1.15)	0.72 (0.44-1.20)	0.44 (0.25-0.80)	0.004
Multivariate OR†	1.00 (ref.)	0.72 (0.41-1.26)	0.74 (0.42-1.29)	0.40 (0.21-0.76)	0.002
Multivariate OR † adjusting for testosterone	1.00 (ref.)	0.69 (0.39-1.22)	0.71 (0.40-1.26)	0.38 (0.20-0.74)	0.001

* Simple conditional logistic regression model adjusting for the matching variables [year of birth, month and year of urine collection, and laboratory batch].

† Multivariate conditional logistic regression models; relative risks were, in addition to matching variables, further adjusted for the following breast cancer risk factors: body mass index (BMI) in six categories (≤21 (ref.), 21.1-23, 23.1-25, 25.1-27, 27.1-30, >30), history of benign breast disease (yes/no), family history (mother or sister) of breast cancer (yes/no), smoking history (never (ref.), past, current), age at menarche in three categories (≤13, 14, 15+), age at menopause in four categories (≤45, 46-49, 50-52, 53+), alcohol consumption per day in grams, three categories (none (ref.), ≤12, >12), years of oral contraceptive use (never (ref.), ≤1 year, >1 year), years of hormone replacement therapy (HRT) use in three categories (never (ref.), ≤1 year, >1 year), parity in three categories (nulliparous (ref.), 1-2, 3+ children), age at first birth in three categories (<25 (ref.), 25-27, ≥28), and participant's educational status in years of schooling, three categories (≤5 years (elementary school, ref.), 8 years (superior education), >8 years).

‡ We tested for trends by modeling aMT6s concentrations continuously and calculating the Wald statistic.

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